An optimized anti-infliximab bridging ELISA for harmonization of anti-infliximab antibody titers in patients with inflammatory bowel diseases

Thomas Van Stappen[*] PharmD, Thomas Billiet[§] MD, Niels Vande Casteele[*] PharmD, PhD, Griet Compernolle[*] Bsc, Els Brouwers[*] Bsc, Séverine Vermeire[§] MD, PhD, Ann Gils[*] PharmD, PhD

[*]Department of Pharmaceutical and Pharmacological Sciences, Laboratory for Therapeutic and Diagnostic Antibodies, KU Leuven, Leuven, Belgium,

[§]Department of Gastroenterology, University hospitals Leuven, Leuven, Belgium.

Correspondence to:
Ann Gils
Laboratory for Therapeutic and Diagnostic Antibodies
Department of Pharmaceutical and Pharmacological Sciences
KU Leuven
O&N II Herestraat 49 - box 820
B-3000 Leuven, Belgium
Tel.: +32 16 32 34 36, Fax: +32 16 32 34 60
E-mail: ann.gils@pharm.kuleuven.be

Conflicts of Interest and Source of Funding:

TVS is a PhD fellow of the Agency for the Promotion and Innovation through Science and Technology in Flanders (IWT, Flanders) and NVC is a Postdoctoral Fellow of the Research Foundation—Flanders (FWO), Belgium; grant number 1260714N. This study was financially supported in part by FWO grant G.0617.12 and grant IOFKP/12/002 from the KU Leuven. NVC received speakers and consultancy fees from MSD, UCB, Janssen Biologics BV and Abbvie. SV received grant support from Centocor, MSD, Abbvie and UCB Pharma. AG has served as a speaker for MSD, Janssen Biologicals, Pfizer and Abbvie and has received Investigator Initiated Research Grants from Pfizer. For the remaining authors no conflicts of interest were declared.

ABSTRACT

Introduction: The formation of anti-infliximab antibodies (ATI) is associated with loss of response and adverse events in patients with inflammatory bowel diseases (IBD), leading to the introduction of ATI monitoring for guiding treatment adjustments. However, a lack of standardization amongst current available assays exists, hampering comparison of results from different studies. This study aimed to improve the harmonization of clinically validated ATI ELISAs by introducing a monoclonal anti-infliximab antibody (MA-IFX).

Methods: A panel of MA-IFX was evaluated as calibrator in the 1st generation ATI ELISA. After selection of one MA-IFX, assay conditions were optimized and biotin-streptavidin enhanced detection of bound infliximab was introduced. The novel 2nd generation ELISA was used for re-analysis of 127 serum samples from a cohort of 12 IBD patients, previously identified as ATI positive.

Results: Out of 55 MA-IFX, MA-IFX10F9 was selected as calibrator in the ATI ELISA. After optimization of the assay conditions, a 4-fold improvement in sensitivity was obtained. Re-analysis of 127 serum samples revealed that in five out of 12 patients (46%) ATI were detected at least one time point earlier with the 2nd generation ELISA compared to the 1st generation ELISA. In one patient, the 2nd generation ELISA allowed to detect ATI before the re-initiation of IFX after a drug holiday.

Conclusions: In addition to the improved sensitivity and specificity of the 2nd generation ATI ELISA, MA-IFX10F9 can serve as a universal calibrator to achieve assay harmonization. Moreover, the superiority of the 2nd generation assay in analyzing serum of re-starters was demonstrated.

Key words: infliximab; Antibodies, Monoclonal; Enzyme-Linked Immunosorbent Assay; immunology; inflammatory bowel diseases

INTRODUCTION

The introduction of biological drugs targeting tumor necrosis factor (anti-TNF), like infliximab (IFX), a chimeric monoclonal antibody, has redefined the treatment of chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases (IBD).[1] Their use led to a considerable improvement in therapy outcome, much needed in clinical practice. However, inherently related to the nature of these drugs, they are often recognized by the immune system as non-self and as a result elicit the formation of anti-drug antibodies (ADA). This immune response leads in numerous cases to loss of response.[2-6] However, in some patients ADA only appear transiently not leading to clinical loss of response, as was observed in a retrospective study with 90 IBD patients on IFX therapy.[7]

The management of patients under biological drugs used to be strictly empirical. Dose adaptations were performed according to the patient's symptoms. Drug was switched in patients with persistent loss of response. With the latest knowledge regarding therapeutic drug monitoring, therapy has become more personalized and efficient.[8] Multiple clinical studies have demonstrated a link between trough concentrations of IFX, which is the drug concentration just before the next dose administration, and clinical response in patients with IBD.[9,10] Low or undetectable trough concentrations correlate with reduced drug efficacy, whilst adequate trough concentrations correlate with sustained response to IFX. Undetectable trough concentrations are often caused by a faster clearance of drug due to complex formation with ADA, hence monitoring of ADA is clinically relevant. Trough concentrations and ADA data were merged into treatment algorithms to aid clinical decision making.[9,11-13]

Different assays exist for measurement of IFX and anti-infliximab antibodies (ATI), but while for drug concentration assays, the drug itself can be used as calibrator, a universal ATI standard doesn't exist. Hence, ATI are reported as arbitrary units towards (monospecific) polyclonal rabbit anti-IFX

antibodies (ELISA[14-16]) or as titration value (ELISA[17], radio-immunoassay[18-20], homogeneous mobility shift assay[21]).

A 1st generation ATI ELISA was developed in the laboratory for Therapeutic and Diagnostic Antibodies (KU Leuven, Leuven, Belgium) using a monospecific rabbit polyclonal anti-IFX (pAb-IFX) calibrator. [14] This assay was clinically validated using sera of IFX treated IBD patients. In a round robin experiment the assay was compared with two other available ATI assays and, although reported values differed and were not interchangeable, a good correlation was found between all three assays.[22] However, the use of a polyclonal antibody calibrator is limited due to batch-to-batch variation inherent to their polyclonal nature.

The aim of this work was to develop a universal calibrator that could be used in different assay formats to harmonize anti-IFX antibody titers in IBD patients. This was done by introducing a monoclonal anti-IFX antibody (MA-IFX) as a calibrator in the current "in-house" developed ATI ELISA and through optimization of the assay conditions. We hypothesized that the newly developed assay could detect ATI earlier resulting from an increased sensitivity. Therefore, a consecutive number of samples were selected from a cohort of IBD patients previously identified to be ATI positive and were re-analyzed with both versions of the assay.

MATERIALS AND METHODS

Bovine serum albumin (BSA), horseradish peroxidase (HRP), polyoxyethylene (20) sorbitan monooleate, sulphuric acid (H_2SO_4), sucrose and citric acid were purchased from Sigma-Aldrich (Steinheim, Germany). EDTA was purchased from Chem-Lab NV (Zedelgem, Belgium) and VWR International (Haasrode, Belgium) delivered disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and sodium chloride (NaCl). Potassium dihydrogen phosphate (KH_2PO_4) and Sulfo-NHS-LC-Biotin were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). HRP-conjugated streptavidin was purchased from IBL International GmbH (Hamburg, Germany). H_2O_2 was purchased

from Merck (Darmstadt, Germany) and o-phenylenediamine and potassium chloride (KCl) from Acros Organics (Geel, Belgium).

IFX was purchased as Remicade from Janssen Biologics B.V. (Leiden, the Netherlands).

Blood samples

Blood samples of patients with IBD were drawn at the infusion unit of the Department of Gastroenterology, UZ Leuven, using the "BD Vacutainer SST II Advance tube" (BD Biosciences, Franklin Lakes, NJ, USA), containing a gel separator and clot activator. After an incubation time of 30 min, serum was prepared by centrifugation (10 min, 1960 *g*, RT) and stored at -20°C.

Introduction of a MA-IFX calibrator in the ATI ELISA

A panel of in-house developed monoclonal anti-IFX antibodies, which was generated, produced and characterized as described in Van Stappen *et al.*[23], was evaluated as calibrator in the 1st generation ATI ELISA.[22] Briefly, 96-well plates were coated overnight with IFX and were blocked with 1% BSA in phosphate buffered saline (PBS; 140 mM NaCl, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 2.7 mM KCl and 1.5 mM KH_2PO_4). Dose-response curve of MA-IFX calibrators were applied on the plate and HRP-labeled IFX was added for detection of bound MA-IFX. Next, plates were developed using 400 µg/mL o-phenylenediamine and 0.003% H_2O_2 in 0.1 M sodium citrate, 0.2 M disodium phosphate buffer pH 5 (citrate buffer) and the reaction was stopped with 4 M H_2SO_4. The absorbance was measured at 490 nm with an EL$_x$808 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) and the MA-IFX dose-response curves were analyzed using nonlinear regression with Graphpad Prism 5.0 (Graphpad Software, San Diego, CA, USA). One MA-IFX was selected for further assay optimization.

Optimization of the ATI assay using biotin-streptavidin enhanced detection

IFX was labeled with Sulfo-NHS-LC-Biotin according to the manufacturer's instructions. IFX was gently mixed with a 20-fold molar excess of biotin solution for 30min at room temperature on a rotator and dialyzed overnight against PBS. The conjugate was sterilized through filtration and stored at 4°C. The

performance of biotin-labeled IFX combined with HRP-labeled streptavidin for detection of bound MA-IFX was evaluated in the ATI ELISA. Briefly, 96-well plates were coated with IFX and free binding places blocked with PBS 1% BSA. Next, a dose-response curve of the selected MA-IFX was applied on the plate, followed by the addition of biotin-labeled IFX as primary conjugate and HRP-labeled streptavidin as secondary conjugate. Plates were developed using o-phenylenediamine and H_2O_2 and the reaction stopped with H_2SO_4. The absorbance was measured at 490 nm and dose-response curves analyzed using nonlinear regression with Graphpad Prism 5.0. Dose-response curves of biotin-labeled IFX were compared to dose-response curves of HRP-labeled IFX. In addition, different assay parameters such as the incubation method, time and temperature and the coating concentration were optimized to maximize the assay performance and minimize the assay execution time.

Lower limit of quantification, accuracy and imprecision

The cut-off value of the assay was determined using 30 anti-TNF naïve IBD patient samples. The samples were applied in a 1:20 dilution and the mean optical density obtained with patient samples plus three standard deviations was compared to the optical density of the MA-IFX10F9 calibrator concentration at 0.078 ng/mL. Accuracy and imprecision were determined by preparing 5 quality control samples of MA-IFX10F9 in PTAE (PBS 0.1% BSA; 0.002% polyoxyethylene (20) sorbitan monooleate; 5 mM EDTA) (5 ng/mL – 10 ng/mL – 20 ng/mL – 50 ng/mL – 100 ng/mL). The quality control samples were diluted 1:20, 1:40, 1:80, 1:160 and six replicates were applied per plate. The experiment was performed in triplicate. All samples were interpolated from the MA-IFX10F9 dose-response curve by nonlinear regression analysis with Graphpad Prism 5.0.

Re-analysis of IBD patient samples

Twelve IBD patients, previously identified as ATI positive using the 1st generation ATI ELISA and among whom nine patients had Crohn's disease and three ulcerative colitis, were selected for re-assessment with the 1st and 2nd generation ATI ELISA. In total, 127 serum samples were analyzed with

a median of 11 (range 5 – 15) consecutive serum samples per patient. Patient samples were interpolated from the dose-response curve using Graphpad Prism 5.0.

Response to treatment

Clinical data were retrospectively collected to determine whether patients were still receiving IFX one year after initial ATI development, with or without a treatment intervention by the treating clinician. An intervention was defined as either surgery, initiation of combo-therapy or a dose adaptation, which included a dose increase of IFX to 10 mg/kg, a shortening of the interval between two drug administrations or a combination of both.

Ethical considerations

This study was approved by the Ethical Committee UZ Leuven in the framework of the Flemish inheritance study for Crohn and colitis (B322201213950/S53684). Informed consent was provided by all patients.

RESULTS

Selection of MA-IFX10F9 for use in the ATI ELISA

The ability of 55 MA-IFX belonging to nine clusters to function as a calibrator in the ATI ELISA setup was evaluated.[23] Based upon the ability to crosslink coated IFX with HRP-labeled IFX, high specificity and high affinity for IFX, MA-IFX10F9 was selected for further use in the ATI ELISA. MA-IFX10F9 is a high affinity, inhibitory IgG1 anti-IFX antibody with κ light chain that binds to the Fab fragment of IFX.

Optimization of the ATI assay using biotin-streptavidin enhanced detection

Using MA-IFX10F9 as a calibrator, biotin-labeled IFX combined with streptavidin-HRP for the detection of bound ATI was compared with HRP-labeled IFX. The calibrator dose-response curve of biotin-streptavidin enhanced ATI detection was found to be more sensitive than HRP-based ATI detection. In addition, different assay parameters were optimized including the incubation method, time and temperature of the different assay steps and the coating concentration, further clarified in

Table 1. Finally, a 4-fold improvement in the sensitivity of the novel 2nd generation ATI ELISA was obtained, when compared to the previously used 1st generation ATI ELISA, shown in Figure 1. The increased sensitivity and the introduction of MA-IFX10F9 as a calibrator allow to report ATI concentrations from patient samples in ng/mL equivalents MA-IFX10F9 instead of µg/mL equivalents pAb-IFX.

Lower limit of quantification, accuracy and imprecision

The use of MA-IFX10F9 as a calibrator in the ATI assay yielded a nonlinear dose-response curve in the range 0.078 ng/mL – 5 ng/mL. The cut-off value of the assay was determined using 30 anti-TNF naïve serum samples. The mean OD of the samples was 0.014 with a standard deviation of 0.008, giving a value of 0.039, which was lower than the OD (0.083) of the lowest point of the calibrator dose-response curve. Taking into account the 1:20 dilution, the assay cut-off was set at 1.6 ng/mL equivalents MA-IFX10F9. The assay complied with the requirements for accuracy (mean recovery ranging from 96% to 102%) and imprecision (coefficient of variation ranging from 7% to 13%) for all quality control samples tested. As 5 ng/mL was still accurately detected with a recovery of 98% and coefficient of variation of 13%, the limit of quantification was set at 5 ng/mL equivalents MA-IFX10F9.

Re-analysis of IBD patient samples

One hundred twenty seven patient samples were re-analyzed with both the 1st and 2nd generation ATI ELISA. Eleven out of 12 ATI positive patients had one or more time points with undetectable IFX concentrations whilst in one patient IFX was detectable in all serum samples.

Nine out of 127 samples (7%) were ATI positive in the 1st generation ELISA and negative in the 2nd generation ELISA. All nine samples came from one patient (patient 12, Figure 2), of which the first sample was an anti-TNF naïve sample. In 57/127 samples (45%) ATI were detected simultaneously with both ELISAs, while 14/127 samples (11%) were ATI positive in the 2nd generation ELISA and negative in the 1st generation ELISA. In 5/12 patients (46%), ATI were detected one or two time

points earlier with the 2nd generation ELISA compared to the 1st generation ELISA (patient 1 to 5, Figure 2). Of those, one patient restarted IFX therapy after a drug holiday of six years and detectable ATI concentrations were found using the 2nd generation ATI ELISA just prior to the first infusion of re-initiation of IFX (patient 5, Figure 2) whereas with the 1st generation ATI ELISA, ATI were only detectable prior to the second infusion of IFX. In Figure 2, the course of ATI formation in all 12 patients is visualized.

Response to treatment

One year after first ATI detection, five out of five patients (patient 1, 2, 4, 8 & 10; Figure 2) with transient ATI were still receiving IFX therapy following an intervention. Three patients (patient 1, 2 & 4) required an interval reduction (from 8 to 4 weeks) and dose intensification (5 mg/kg to 10 mg/kg) to overcome the ATI response. In addition, patient 2 received prophylactic therapy and addition of an immunosuppressant. Patient 8 only required interval shortening from 8 to 7 weeks. Patient 10 underwent a right hemicolectomy, allowing him/her to continue IFX therapy without interval shortening or dose adaptation. All patients who persistently developed ATI lost clinical response to IFX and were consequently switched to other (anti-TNF) drugs, five out of six within one year after first ATI detection. Of those, one patient suffered from an acute infusion reaction at the second infusion of re-initiation of IFX after a drug holiday of six years. The patient wherein no ATI were detected with the 2nd generation ATI ELISA still received IFX after one year and was doing clinically well.

DISCUSSION

The measurement of ADA is challenging and can be performed with many different techniques (i.e. ELISA[14-17], radio-immunoassay[18-20], homogeneous mobility shift assay[21]). Bridging ELISA's and radio-immunoassays are commonly used, but have the disadvantage that ADA cannot be detected in the presence of drug. Drug sensitivity can be reduced using sample pretreatment steps such as acid dissociation, which is used for instance in the pH-shift-anti-idiotype antigen binding test (PIA).[19]

Nevertheless, whether the measurement of ADA in complex with drug is clinically relevant is much debated. In a recent study in 99 rheumatoid arthritis patients treated with adalimumab, the detection of anti-adalimumab antibodies (ATA) in complex with adalimumab using the PIA assay was not linked to a reduction in clinical response, while free ATA measured with the drug sensitive antigen binding test was correlated.[24] However, the likelihood of patients with complexed ATA to have detectable free ATA during subsequent follow-up was higher than for patients without complexed ATA. Furthermore, the clinical equivalence of different assays was investigated in a post-hoc analysis of a randomized controlled trial.[25] In this study it was seen that different assays, including a drug sensitive homogeneous mobility shift assay, did not largely differ in predicting the best treatment strategy at time of loss of response to IFX therapy.

Besides assay dependent shortcomings and their clinical relevancy, a major issue currently acknowledged by experts is the fact that different techniques are not reporting ATI in the same unit. While some assays express ATI as arbitrary units towards an antibody calibrator (ELISA[14-16]), other assays make use of titration values (ELISA[17], radio-immunoassay[18-20], homogeneous mobility shift assay[21]), inhibiting a direct comparison of reported ATI values between different assays.[22,26] Harmonization of ATI assays will be indispensable to facilitate and generalize clinical decision making at time of loss of response to IFX. Comparison studies aiming to define assay conversion factors and universal calibrators may serve as a stepping stone to this ambitious target.

Polyclonal anti-IFX antibodies as calibrator are not first choice as they cross-react with other anti-TNF drugs and introduce variability due to batch-to-batch variation inherent to their polyclonal nature. The introduction of a highly specific monoclonal anti-IFX antibody, MA-IFX10F9, offers the advantage of continuous production, thereby limiting batch-to-batch variation. In addition, the initial protocol of the 1st generation ATI ELISA was optimized to improve the assay speed, performance and specificity. Furthermore, titration discrepancies frequently observed with the 1st generation ATI ELISA are reduced with the 2nd generation ATI ELISA.

The improved assay sensitivity of the 2nd generation ATI ELISA resulted in an earlier detection of ATI in 5/12 patients (46%) compared to the 1st generation ELISA. One of these patients restarted IFX therapy after a drug holiday of six years and the 1st generation ATI ELISA detected ATI only prior to the second infusion of IFX after re-initiation, during which the patient developed an acute infusion reaction. The 2nd generation ATI ELISA detected ATI already prior to the first infusion of re-initiation of IFX. This example clearly demonstrates the superiority of the 2nd generation ELISA in patients restarting IFX after a drug holiday.

Our results also indicate an enhanced assay specificity since one patient was reclassified as ATI negative with the 2nd generation ELISA that was initially classified as ATI positive with the 1st generation ELISA. This patient was found to have detectable IFX trough concentrations over the course of time, most likely indicative of an aspecific response in the 1st generation ELISA.

Retrospectively, it was seen that five out of five patients with transient ATI still received IFX one year after the first ATI detection, while IFX treatment was discontinued in all patients with persistent ATI development, five out of six within one year after initial ATI detection – reconfirming previous findings that ATI development may be overcome upon an intervention and not necessarily lead to clinical loss of response.[7,24] Follow-up of ATI in consecutive serum samples is therefore advised.

In conclusion, we have introduced MA-IFX10F9, a monoclonal anti-IFX antibody that can serve as a universal calibrator to achieve harmonization of ATI titers. In addition, we have increased the sensitivity and specificity of the ATI ELISA resulting in an earlier detection of ATI formation in a considerable amount of ATI positive IBD patients. Early ATI detection can improve timely treatment decisions to prevent loss of response and allows choosing the most cost-efficient treatment option in case of loss of response. In patients restarting IFX after a drug holiday, ATI measurements are crucial to avoid the risk of adverse events such as infusion reactions and unnecessary treatment costs.

ACKNOWLEDGMENTS

We gratefully thank Annelien Grevendonck for her aid in conducting the experiments.

REFERENCES

1. Rutgeerts P, Vermeire S, Van Assche G. Biological therapies for inflammatory bowel diseases. Gastroenterology. 2009;136:1182-1197.

2. Baert F, Noman M, Vermeire S, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N Engl J Med. 2003;348:601-608.

3. Yanai H, Hanauer SB. Assessing response and loss of response to biological therapies in IBD. Am J Gastroenterol. 2011;106:685-698.

4. Nanda KS, Cheifetz AS, Moss AC. Impact of antibodies to infliximab on clinical outcomes and serum infliximab levels in patients with inflammatory bowel disease (IBD): a meta-analysis. Am J Gastroenterol. 2013;108:40-47.

5. Karmiris K, Paintaud G, Noman M, et al. Influence of trough serum levels and immunogenicity on long-term outcome of adalimumab therapy in Crohn's disease. Gastroenterology. 2009;137:1628-1640.

6. Steenholdt C, Svenson M, Bendtzen K, et al. Severe infusion reactions to infliximab: aetiology, immunogenicity and risk factors in patients with inflammatory bowel disease. Aliment Pharmacol Ther. 2011;34:51-58.

7. Vande Casteele N, Gils A, Singh S, et al. Antibody response to infliximab and its impact on pharmacokinetics can be transient. Am J Gastroenterol. 2013;108:962-971.

8. Vande Casteele N, Feagan BG, Gils A, et al. Therapeutic drug monitoring in inflammatory bowel disease: current state and future perspectives. Curr Gastroenterol Rep. 2014;16:378.

9. Maser EA, Villela R, Silverberg MS, et al. Association of trough serum infliximab to clinical outcome after scheduled maintenance treatment for Crohn's disease. Clin Gastroenterol H. 2006;4:1248-1254.

10. Seow CH, Newman A, Irwin SP, et al. Trough serum infliximab: a predictive factor of clinical outcome for infliximab treatment in acute ulcerative colitis. Gut. 2010;59:49-54.

11. Ordas I, Mould DR, Feagan BG, et al. Anti-TNF monoclonal antibodies in inflammatory bowel disease: pharmacokinetics-based dosing paradigms. Clin Pharmacol Ther. 2012;91:635-646.

12. Vande Casteele N, Compernolle G, Ballet V, et al. OP11 Individualised infliximab treatment using therapeutic drug monitoring: A prospective controlled Trough level Adapted infliXImab Treatment (TAXIT) trial. J Crohns Colitis. 2012;6:S6.

13. Vermeire S, Gils A. Value of drug level testing and antibody assays in optimising biological therapy. Frontline Gastroenterol. 2013;4:41-43.

14. Vande Casteele N, Ballet V, Van Assche G, et al. Early serial trough and antidrug antibody level measurements predict clinical outcome of infliximab and adalimumab treatment. Gut. 2012;61:321.

15. Ben-Horin S, Yavzori M, Katz L, et al. The immunogenic part of infliximab is the F(ab')2, but measuring antibodies to the intact infliximab molecule is more clinically useful. Gut. 2011;60:41-48.

16. Ternant D, Aubourg A, Magdelaine-Beuzelin C, et al. Infliximab pharmacokinetics in inflammatory bowel disease patients. Ther Drug Monit. 2008;30:523-529.

17. Candon S, Mosca A, Ruemmele F, et al. Clinical and biological consequences of immunization to infliximab in pediatric Crohn's disease. Clin Immunol. 2006;118:11-19.

18. Wolbink GJ, Vis M, Lems W, et al. Development of antiinfliximab antibodies and relationship to clinical response in patients with rheumatoid arthritis. Arthritis Rheum. 2006;54:711-715.

19. van Schouwenburg PA, Bartelds GM, Hart MH, et al. A novel method for the detection of antibodies to adalimumab in the presence of drug reveals "hidden" immunogenicity in rheumatoid arthritis patients. J Immunol Methods. 2010;362:82-88.

20. Bendtzen K, Geborek P, Svenson M, et al. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. Arthritis Rheum. 2006;54:3782-3789.

21. Wang SL, Ohrmund L, Hauenstein S, et al. Development and validation of a homogeneous mobility shift assay for the measurement of infliximab and antibodies-to-infliximab levels in patient serum. J Immunol Methods. 2012;382:177-188.

22. Vande Casteele N, Buurman DJ, Sturkenboom MG, et al. Detection of infliximab levels and anti-infliximab antibodies: a comparison of three different assays. Aliment Pharmacol Ther. 2012;36:765-771.

23. Van Stappen T, Brouwers E, Tops S, et al. Generation of a highly specific monoclonal anti-infliximab antibody for harmonization of TNF-coated infliximab assays. Ther Drug Monit. 2014; *in press*.

24. van Schouwenburg PA, Krieckaert CL, Rispens T, et al. Long-term measurement of anti-adalimumab using pH-shift-anti-idiotype antigen binding test shows predictive value and transient antibody formation. Ann Rheum Dis. 2013;72:1680-1686.

25. Steenholdt C, Bendtzen K, Brynskov J, et al. Clinical implications of measuring drug and anti-drug antibodies by different assays when optimizing infliximab treatment failure in Crohn's disease: post hoc analysis of a randomized controlled trial. Am J Gastroenterol. 2014;109:1055-1064.

26. Steenholdt C, Ainsworth MA, Tovey M, et al. Comparison of techniques for monitoring infliximab and antibodies against infliximab in Crohn's disease. Ther Drug Monit. 2013;35:530-538.

27. Steenholdt C, Brynskov J, Thomsen OO, et al. Individualised therapy is more cost-effective than dose intensification in patients with Crohn's disease who lose response to anti-TNF treatment: a randomised, controlled trial. Gut. 2014;63:919-927.

FIGURE LEGENDS

FIGURE 1. Dose-response curves using the pAb-IFX calibrator in the 1st generation ATI ELISA (left) and the MA-IFX10F9 calibrator in the 2nd generation anti-infliximab antibody ELISA (right). pAb-IFX, polyclonal anti-infliximab antibody; ATI, anti-infliximab antibodies; MA-IFX, monoclonal anti-infliximab calibrator; OD, optical density

FIGURE 2. Re-analysis of inflammatory bowel diseases patient samples using the 1st (dashed line, left y-axis) and 2nd generation (solid line, right y-axis) ATI ELISA. ATI concentrations are reported in µg/mL equivalents pAb-IFX and ng/mL equivalents MA-IFX10F9 in the 1st and 2nd generation ATI ELISA, respectively. IFX concentrations are reported in µg/mL (dotted line, left y-axis). Time points marked with an asterisk indicate clinical interventions and double asterisks unavailability of sample. In total, 71/117 samples (61%) were found ATI positive in the 2nd generation ELISA, while the 1st generation ELISA detected ATI in only 57/117 (49%). In patients 1 to 5, first ATI detection was seen one or two time points earlier with the 2nd generation ELISA than with the 1st generation ELISA (arrows). Of those, patient 5 was reinitiated with IFX after a drug holiday of 6 years, despite detectable ATI at restart using the 2nd generation ATI ELISA (arrow). In patient 12, ATI were detected in multiple consecutive samples with the 1st generation ELISA whereas the 2nd generation ELISA did not detect ATI. ATI, anti-infliximab antibodies; pAb-IFX, polyclonal anti-infliximab antibody; MA-IFX, monoclonal anti-infliximab antibody; IFX, infliximab.